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Thiamethoxam impairs honey bee visual learning, alters decision times, and increases abnormal behaviors

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ABSTRACT

Learning is important for honey bee fitness and the pollination services that they provide. Neonicotinoid pesticides impair learning, fitness, colony health, and pollination, but most studies on how they affect bee learning have focused on olfactory learning. We tested the effects of field realistic doses of 0.8 ng/bee and 1.34 ng/bee of the neonicotinoid pesticide, thiamethoxam (TMX), on bee visual learning. We adapted a T-maze bioassay and classically conditioned bees to associate sugar reward with a simulated flower color (blue or yellow light) in a choice assay. At 1.34 ng/bee, TMX significantly reduced correct choices in the final learning trial as compared to the control treatment. There was no TMX effect in our 1-h memory test. We found stronger effects on decision time and abnormal behaviors. TMX decreased bee decision times, a potential byproduct of induced hyperactivity since bees walked to make choices. Behaviors (falling, trembling, and rapid abnormal movements) were significantly increased by both TMX doses as compared to the control treatment. These results suggest that the effects of neonicotinoids on bee visual learning should be further studied and incorporated into Risk Assessment protocols.

1. Introduction

Given the widespread use of neonicotinoids, a class of insecticides that block nicotinic acetylcholine receptors (Tosi and Nieh, 2017) and stimulate cholinergic neurons (Johnson et al., 2010), concern is growing about their effects on pollinators such as the western honeybee, *Apis mellifera* (Sanchez-Bayo and Goka, 2014). Honey bees can provide important pollination services for natural and agricultural ecosystems (Hung et al., 2018; Winfree et al., 2011) and their pollination efficacy is enhanced by visual and olfactory learning. Bees learn the appearance and odors of rewarding food to improve their searches for the same floral species throughout the landscape (Dukas and Visscher, 1994). Honey bees therefore have excellent visual and olfactory memories (Avargues-Weber and Mota, 2016; Matsumoto et al., 2012). However, multiple studies have now demonstrated that neonicotinoid pesticides can harm honey bee (*A. mellifera*) learning and foraging (Decourtye et al., 2013; Han et al., 2010), impairing colony fitness and likely reducing their ability to pollinate (Lundin et al., 2015).

Exposure to even small, field-realistic doses of imidacloprid can harm olfactory *A. mellifera* learning (Yang et al., 2012). Some studies show that neonicotinoids can decrease olfactory short-term memory formation in foragers but leave long-term memory unaffected (Wright et al., 2015). Other research demonstrates that both olfactory short-term and long-term memory can be harmed (Williamson and Wright, 2013).

However, relatively few studies have examined the effects of neonicotinoids on honey bee visual learning. A homing study of free-flying bees showed that neonicotinoids impaired their abilities to use landmarks and return to their nest, suggesting visual learning impairment (Fischer et al., 2014). Han et al. (2010) tested pesticide effects on visual learning and reported that honey bees fed pollen with imidacloprid had impaired color learning in a T-maze. This relative lack of studies testing if pesticides affect visual learning may arise because honey bee visual learning is more robust when conducted with unrestrained, freely flying bees (Avargues-Weber and Mota, 2016). Such tests are typically more challenging to conduct than the laboratory assays used to study olfactory learning. Reliable laboratory assays of visual learning are therefore desirable because they enable easier testing of larger numbers of bees under controlled conditions that do not depend upon favorable weather (Avargues-Weber and Mota, 2016). The development and implementation of risk assessment protocols by pesticide regulatory agencies would thus be facilitated with such assays (Tosi and Nieh, 2019).

We focused on thiamethoxam (TMX), a neonicotinoid, that is used on a wide range of crops, is one of the top three neonicotinoids used in

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the United States since 2012 (Bass et al., 2015), and is one of five neonicotinoid pesticides increasingly detected in domestic and imported food since 2014 (Craddock et al., 2019). The major breakdown product of TMX, clothianidin, impairs honey bee memory (Tison et al., 2019) and is highly toxic to insects (Nauen et al., 2003), which contributes to the long-term environmental hazards of TMX (Kah et al., 2018). Our goals were to test the effects of thiamethoxam on honey bee visual learning and refine the lab-based T-maze assay of Han et al. (2010) to improve its utility as a Risk Assessment tool. Finally, we wished to further explore the effects of TMX on bee behavior because thiamethoxam can impair honey bee locomotion (Tosi and Nieh, 2017), and other nicotinic acetylcholine receptor agonists can increase the number of abnormal bee behaviors (Tosi and Nieh, 2019). We therefore hypothesized that thiamethoxam would increase abnormal honey bee behaviors in the T-maze and reduce color learning and color memory.

2. Materials & methods

2.1. Study site and colonies

Experiments were conducted with ten honey bee colonies housed in standard Langstroth hive boxes in Biology Field Station apiary (32°53′07.9″N 117°13′55.1″W) at the University of California San Diego, La Jolla, California, USA. Colonies were healthy as determined by standard inspection techniques (Dietemann et al., 2013). Bees were tested in a dark, temperature-controlled room (30 °C and 40% relative humidity). We used this higher room temperature because preliminary experiments showed that these conditions increased the motivation of bees to consume the sucrose reward.

2.2. Ethics statement

All bees were treated accordingly to standard ethical guidelines (Dietemann et al., 2013) in an apiary that is registered with the County of San Diego and meets the requirements of the County and California Food and Agricultural Code Sections 29101, 29040, and 29070. Control bees were released back to their colonies, but pesticide-treated bees were euthanized at 0 °C to avoid contaminating colonies.

2.3. Visual learning apparatus

We modified the T-maze bioassay developed by Han et al. (2010) to test the effects of pesticides and GM products on *A. mellifera* visual learning and behavior. We used LED lights as the visual stimulus instead of filtered white light (Han et al., 2010) because LED light intensity can more easily be adjusted and equalized. The intensity of these lights (CO-RODE Amazon; yellow: 589–591 nm; blue: 460–465 nm) was adjusted to 120 lux, as measured with a digital illuminance meter (DrMeter, LX1220B) for blue and yellow light shining through the maze tube (Fig. 1A). The maze consisted of clear plastic tubes (FORMUFIT P001FGP-UV-5 schedule 40 clear PVC pipe, furniture grade, 2.54 cm outer diameter, 2.5cm inner diameter, and matching clear F001TEE-UV T fittings). Lights were mounted on a breadboard with 12 blue and 12 yellow lights alternating along each side. Each side had a switch that controlled the color turned on. Bees were randomly selected for reward conditioning to either blue or yellow light. The sides for these colors were randomly determined at the start of the trials and the side associated with the rewarded color was pseudo-randomly alternated throughout the trials (see below).

Foragers were collected (captured in a clear plastic cage, 11 × 9 × 11 cm length × width × height, approximately 25 bees per cage) from a 2.0 M sucrose feeder (prepared with analytical grade sucrose with Milli-Q water) placed at the colony entrance. They were group-fed 2.0 M sucrose to satiation (two 5.0 ml syringes provided per cage), and then incubated overnight (32.5 °C and 60% relative humidity) to equilibrate their hunger states (Avargues-Weber and Mota, 2016). Bees were then selected at random, each placed inside a separate scintillation vial with a perforated cap and individually fed the treatments by the experimenter with a Gilson micropipette: 2 μl of 50% pure sucrose solution (w/w) or 2 μl of 50% sucrose solution containing either 0.8 ng or 1.34 ng TMX. The researcher visually verified that all bees consumed the full dose. These bees were then incubated in the dark (32.5 °C and 60% humidity) for 1 h (Tosi and Nieh, 2017).

2.4. Pesticide doses

We exposed bees to an acute dose of 0.8 ng TMX/bee (low dose) or 1.34 ng TMX/bee (higher dose). The low and higher dose experiments were run sequentially. Both doses are sublethal and field realistic (Henry et al., 2012; Tosi et al., 2017; Tosi and Nieh, 2017). The European Food Safety Authority (EFSA) estimated that foragers can consume up to 1.80 ng TMX/bee in 1 h of foraging for nectar (10% sugar w/w, oilseed rape contaminated with 15 ppb of TMX (EFSA, 2012; Tosi and Nieh, 2017). In addition, foragers can imbibe up to 6.66 ng TMX/bee/day while collecting nectar (with 5 ppb of TMX) from TMX seed-treated plants such as oilseed rape (EFSA, 2012). Our higher dose of 1.34 ng TMX/bee is therefore closer to a worst-case scenario, whereas the lower dose of 0.8 ng TMX/bee represents a more common field-realistic exposure.

We prepared a 200 ppm TMX stock solution with Milli-Q water and analytical grade 99.3% purity TMX (CAS#153719-23-43, Sigma Aldrich 37924-100 MG-R) with an analytical lab balance. No acetone was used, and the TMX completely dissolved. This solution was kept in darkness (Eppendorf tubes covered with aluminum foil), frozen, and defrosted at 4 °C when used. Serial dilutions were made with Milli-Q water and reagent grade 50% sucrose solution (also made with Milli-Q water). All researchers were blind to the identity of the solutions being used. Solution identity was only revealed after all data had been collected.

2.5. Maze learning procedure

To help improve learning, we used a learning pre-trial (method of Dobrin and Fahrbach, 2012) in which bees learned to associate the rewarded color with a food reward. Bees were introduced to the entrance of the apparatus under dim red light (which they have difficulty seeing). Per bee, we randomly selected which color would be the conditioned stimulus (CS). When the bee reached the maze arm with the CS (no other color was turned on), it was rewarded with a 2.0 M sucrose solution for 3 s of feeding, recaptured in the same vial it was released in, and returned to the dark.

We then trained bees with six learning trials in which they had to discriminate between two different light colors (10 min intertrial intervals). When the bee entered the T-maze apparatus, both blue and yellow lights were simultaneously turned on, at opposite ends of the maze (Fig. 1A). One color was the CS that would be associated with sucrose reward. The other was the non-rewarded stimulus. The side for the CS was randomly chosen for the first trial and then alternated in a pseudorandom sequence (different randomly chosen sequences for which each color was always shown for three times on each side because we had six learning discrimination trials). A bee was only rewarded if it reached the CS color (score = 1), defined as passing a decision boundary that was 5 cm from the midpoint of the maze junction (dashed lines in Fig. 1A). A bee that made the incorrect decision and chose the unrewarded light color (score = 0) had the incorrect color light turned off after 5 s and therefore went to the correct light (basic phototaxis) where it was rewarded. Decision time was measured starting when a bee entered the apparatus and ended once it passed a decision line (Fig. 1A). Bees generally did not make a choice after 5 min, and thus a bee that took longer than 5 min was recording as having failed. This bee was gently captured in a vial and then released and rewarded at the correct side and color.

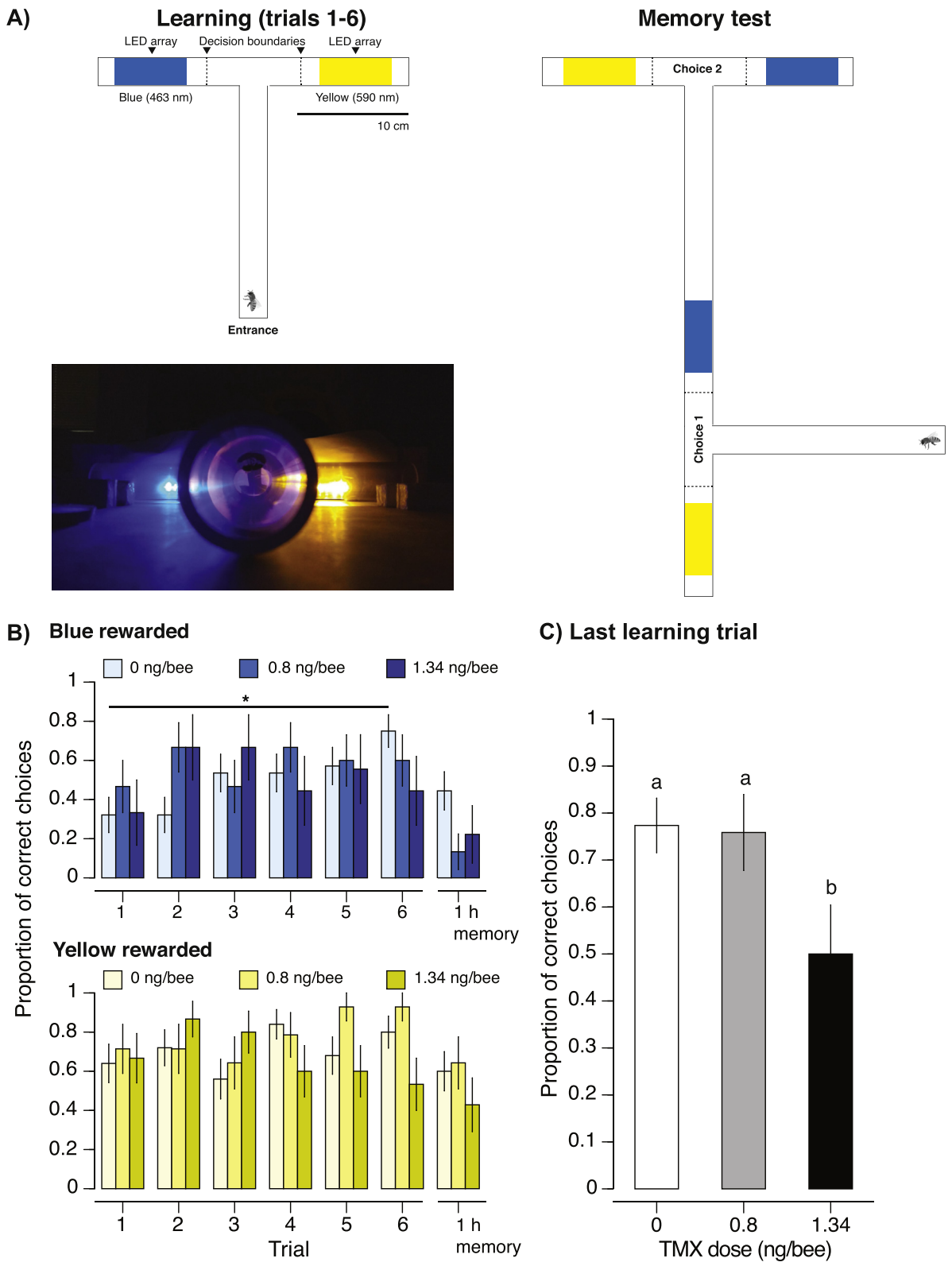

Fig. 1. Effects of TMX on visual learning of color. (A) The design of the apparatus is shown to scale for the learning trials (1–6) and the 1 h memory test in which bees had to negotiate two arms of the maze (choice 1 and then choice 2). Dashed lines indicate decision boundaries. The photo shows a bee inside the maze. (B) The proportion of correct choices made by bees in all learning trials and the 1 h memory test per reward color. The horizontal bar (*) shows significant learning in trial 6 vs. trial 1 for control bees (Tukey HSD test, $P < 0.05$). (C) In the last learning trial (trial 6), there was no effect of rewarded color, and thus the data were pooled. Different letters indicate significant differences (Tukey HSD test, $P < 0.05$). Means and standard errors are shown in all plots.

Table 1
Abnormal behaviors of honey bees in the maze.

Monitored Activity	Description
Falling	A bee falling and turning onto its dorsum. Bees could often right themselves and resume walking normally after a fall. Each bee could therefore fall multiple times per trial, and all falls were counted.
Trembling	A bee continuously stumbling and rapidly vibrating its body while walking. Because this behavior generally lasted the entire trial, it was scored only once per trial.
Hyperactivity	Rapid abnormal movements (bee moved anomalously in multiple directions, not in a straight line) and rolling (bee rapidly and continuously turning and spinning, usually because of loss of limb coordination).

Bees that did not feed for two trials or failed to complete the maze within 5 min per trial over two trials were excluded from our analyses because they demonstrated little motivation to accept the reward or to complete the learning experiment (Bitterman et al., 1983). Because pesticide could have contributed to these failures, we also analyzed the failure data.

One hour after these learning trials, we performed a memory retention test in which bees had to sequentially navigate two T-mazes, with the second attached to the first (Fig. 1A). Each bee also had only 5 min to complete this trial. The memory test was unrewarded and a bee was only considered to have made a correct choice (score = 1) if it correctly navigated both mazes. Incorrectly navigating either of the two mazes was scored as incorrect (score = 0).

2.6. Abnormal behaviors

Three abnormal behaviors were scored during the experiment: falling, trembling, and hyperactivity. These are defined and described in detail in Table 1 and were chosen because they were common and similar to those reported by Tosi and Nieh (2017) whose also studied the effects of TMX on *A. mellifera*.

2.7. Statistics

To determine if pesticide affected the proportion of bees that successfully completed their trials or died during trials, we ran two-tailed Fisher's Exact 2 × 3 tests (http://vassarstats.net/fisher2x3.html) to compare between the three treatment levels (0, 0.8, and 1.34 ng TMX/bee).

We used JMP v. 13.0 statistical software and ran Repeated Measures Mixed Models (REML algorithm) to test the effects of pesticide (Matsumoto et al., 2012). We only included bees that completed all trials. This excluded 53 bees (33%) from the analyses, but allowed us to compare learning between all trials with a balanced sample size. We did not exclude bees based upon their learning performance. For each of these models, we used the following fixed effects (trial and dose) and random effects (colony and bee identity). For all models, we first tested all fixed effect interactions and then eliminated non-significant interactions.

For visual learning, we first ran a separate model for each color and then made comparisons with Tukey Honestly Significant Difference (HSD) tests because Han et al. (2010) demonstrated that *A. mellifera* foragers have an innate preference for yellow in their T-maze assay. Based upon visual inspection of the data graphs, we then examined only learning in the sixth and final learning trial (Mixed Model with colony as a random effect and dose as a fixed effect). We first tested the effect of color but pooled the data from both colors because color was not significant. For our memory models, we only examined a single time point per bee and therefore ran a Mixed Model separately for each color, with colony as a random effect. For decision times and abnormal behaviors, we used Repeated Measures Mixed Models (see above) with both colors pooled because the first model showed no significant effect of color. To test the potential effect of decision time (speed) on correct choices (accuracy), we also used a Repeated Measures Mixed Models with decision time, TMX treatment, trial, rewarded color, and all interactions as fixed effects (colony as random effect). We used Tukey HSD tests (all pairwise comparisons) and LS Means Contrast post-hoc tests (limited comparisons) to make corrected pairwise comparisons, with the choice of test determined by visual inspection of the data.

2.8. Data accessibility statement

All data are available on Zenodo.org at DOI 10.5281/zenodo.3672157.

3. Results

In total, we used ten colonies and analyzed the data from 108 bees: 29 bees in the 0.8 ng/bee trials (low dose), 24 bees in the 1.34 ng/bee trials (higher dose), and 55 bees in control experiments. Temperature and humidity respectively averaged 29.6 °C ± 1.8 °C and 43.2% ± 8.3% (Mean ± SD).

Pesticide did not affect (Fisher's Exact 3 × 2 test, $P = 0.458$) the percentage of bees that completed their learning trials: control (63%), 0.8 ng/bee (88%), and 1.34 ng/bee (55%). Our pesticide treatments were sublethal and did not affect survival during the experiment: control (99% survived), 0.8 ng/bee (97%), and 1.34 ng/bee (100%).

3.1. Bee visual learning

As expected, bees learned blue but not yellow (Fig. 1B). For blue as the CS, they exhibited significant learning because correct responses were higher in trial 6 than in trial 1 (Tukey HSD test, $P < 0.05$). They did not significantly learn when yellow was the CS color (trial 1 vs. 6, Tukey HSD test, $P < 0.05$).

Given the high variation in bee choices, we decided to take a simpler approach and just compare choice in the sixth and final learning trial. In this final learning trial, there were no significant effects of color ($F_{1,96} = 2.84$, $P = 0.095$) or the interaction color x dose ($F_{2,5} = 0.78$, $P = 0.51$). However, there was a significant effect of dose ($F_{2,58} = 4.33$, $P = 0.018$; Fig. 1C) such that higher dose bees had a significantly lower proportion of correct choices as compared with control and low dose bees (Tukey HSD test, $P < 0.05$). Colony accounted for < 1% of model variance. Pesticide dose did not affect bee choices in the 1 h memory test for either blue ($F_{2,42} = 1.80$, $P = 0.18$) or yellow ($F_{2,42} = 0.59$, $P = 0.56$) as the CS color (colony accounted for < 4% of model variances, Fig. 1B).

3.2. Decision time

Bees learned the navigate the maze more rapidly over multiple trials. There was no significant effect of CS color on decision time ($F_{1,100} = 0.20$, $P = 0.659$), and thus we pooled data from both colors. Decision times significantly decreased with trial (trial effect: $F_{5,525} = 7.15$, $P < 0.0001$; Fig. 2). In addition, control bees took significantly longer to make decisions than higher dose bees ($F_{2,83} = 5.23$, $P = 0.0073$; Fig. 2). The interaction dose x trial was not significant ($F_{10,515} = 0.80$, $P = 0.632$). In trials 1, 2, 4, and 5, control bees took significantly longer than pesticide-treated bees (LS Means Contrast tests, $F_{1,358} \geq 4.40$, $P \leq 0.037$). Colony accounted for 3% of

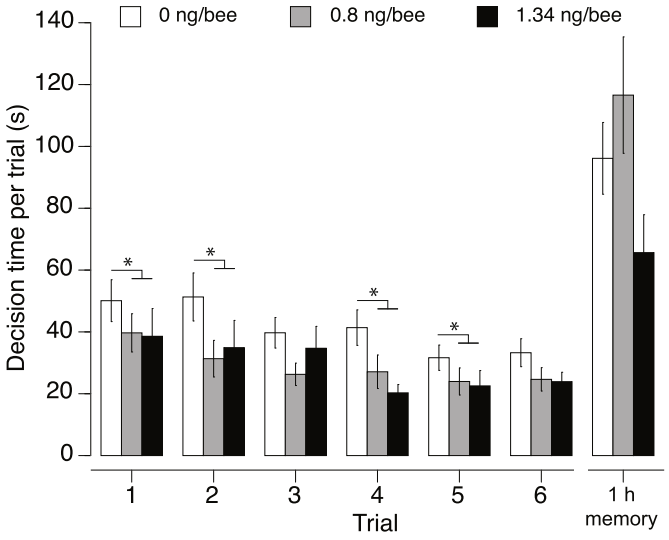

Fig. 2. Effect of TMX on the average time that each bee took to make a decision per learning trial and in the 1 h memory test. Bars and asterisks show the results of significant LS Means Contrast test ($P \leq 0.037$) between the control treatment (0 ng/bee) and both pesticide treatments. Data from both reward colors are pooled because there was no significant effect of color. Means and standard errors are shown.

model variance.

The likelihood of correct choices was not significantly predicted by decision time ($F_{1,550} = 0.02$, $P = 0.90$), trial ($F_{5,527} = 2.12$, $P = 0.06$), or TMX treatment ($F_{2,87} = 0.75$, $P = 0.47$). However, there was a significant effect of rewarded color ($F_{1,101} = 18.00$, $P < 0.0001$) because bees have an innate preference for yellow. No interactions were significant ($F_{10,535} \leq 1.42$, $P \geq 0.17$) and colony accounted for less than 4% of model variance.

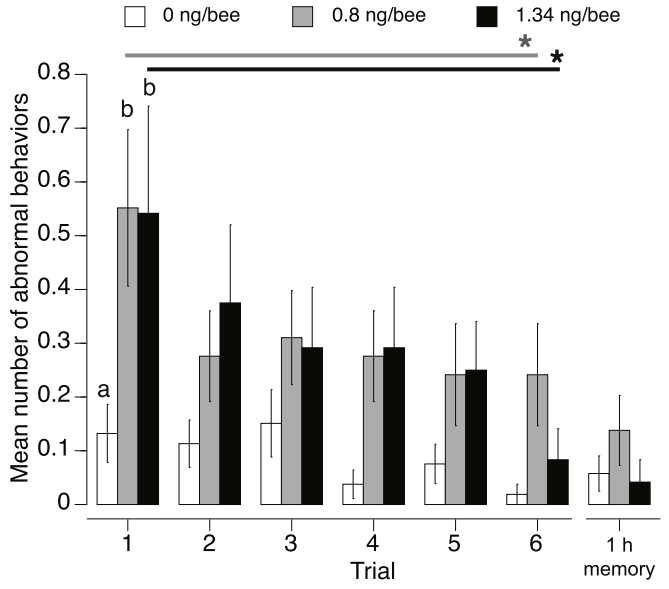

Fig. 3. Effect of TMX on the total number of abnormal behaviors (Table 1) per trial. Different letters show significant differences (Tukey HSD test, $P < 0.05$). In addition, the horizontal bars respectively show that abnormal behaviors significantly declined for the 0.8 ng/bee dose (gray bar *) and the 1.34 ng/bee dose (black bar *) in trial 6 as compared to trial 1 (Tukey HSD test, $P < 0.05$). Means and standard errors are shown.

3.3. Abnormal behaviors

Pesticide increased the number of abnormal behaviors (Fig. 3). Specifically, in trial 1, both low and higher dose bees had significantly more abnormal behaviors than control bees (Tukey HSD test, $P < 0.05$). There was a significant effect of trial because abnormal behaviors decreased over time (trial effect, $F_{6,629} = 11.82$, $P < 0.0001$). There were also significant effects of dose ($F_{2,102} = 4.17$, $P = 0.018$), and the interaction trial x dose ($F_{12,629} = 2.44$, $P = 0.0042$) on abnormal behaviors. There were no significant effects of dose in any other trial (Tukey HSD test, $P > 0.05$). Colony accounted for 13% of total model variance.

4. Discussion

In general, we found weak effects of TMX on bee visual learning. However, bees learned to associate blue light with a sucrose reward: control bees demonstrated improvement in the final learning trial (trial 6 vs. trial 1, Fig. 1B). Bees did not exhibit any significant learning with yellow light as the reward, as expected (Han et al., 2010). The higher pesticide dose (1.34 ng/bee) decreased correct choices in the final learning trial as compared to the control or lower pesticide dose (0.8 ng/bee) treatments (Fig. 1C). Memory was not affected by pesticide treatment. Stronger effects of TMX were revealed by examining bee decision times and abnormal behaviors. Bees fed TMX had faster decision times, perhaps reflecting a hyperactive state that can be induced by TMX (Tosi and Nieh, 2017). Abnormal behaviors, particularly falling, occurred at a significantly higher rate in TMX treated bees (both doses) than in control bees, and there were more abnormal behaviors in earlier trials, likely reflecting changing pesticide effects and behavioral recovery over time.

4.1. Visual learning

The failure of our bees to learn the yellow light is not surprising because the strong preference of bees for yellow (Han et al., 2010; Zhang, 1996) appears in their first trial, in which 64% of our control bees chose the yellow arm. Han et al. (2010) only trained bees to associate blue light with reward to demonstrate that bees could associate a *non-preferred* color with a reward. In our study, we explored potential learning for yellow as the CS, but, perhaps predictably, we found no significant learning of yellow because the highest proportion of choice for yellow light (68%) was only marginally higher than the 64% choice for yellow shown in the first trial. In contrast, bees with blue as the CS showed an increase from 32% entering the blue arm of the maze (approximately chance level) in the first trial to 75% blue choice in the final learning trial (a significant 43% increase).

In our final learning trial, we were able to find a significant (but albeit weak) effect of TMX: higher dosed bees (1.34 ng TMX/bee) demonstrated significantly fewer correct choices than control or low dosed bees. This result agrees with prior research that TMX can reduce honey bee olfactory short-term memory (Wright et al., 2015) and that neonicotinoids can harm visual learning (Han et al., 2010). Because bees generally learn better in free-flying visual assays than in restrained or even lab-based walking assays of visual learning (Avargues-Weber and Mota, 2016), this lack of a strong TMX effect on visual learning could have arisen from the limitations of our assay. However, neonicotinoids can impair olfactory learning without significantly harming visual learning. Imidacloprid, thiamethoxam, and clothianidin (a metabolite of thiamethoxam) did not impair the ability of free flying bumblebees to form visual associations (Muth et al., 2019).

4.2. Decision time

Bees significantly decreased their decision times as the trials progressed (Fig. 2), likely because they became more familiar and learned

how to better navigate the apparatus over time. Higher dose bees also completed their trials significantly more rapidly than control bees. Similarly, Tosi et al. (2017) found that TMX dosed bees took less time to reach the top of a vertical phototaxis arena, perhaps because of pesticide-induced hyperactivity (Tosi and Nieh, 2017). Our pairwise comparisons likewise show that dosed bees were faster than control bees (Fig. 2).

It is relevant to consider potential speed-accuracy tradeoffs. In *Bombus terrestris,* foraging bees sacrifice the accuracy of their destination for speed and vice versa (Chittka et al., 2003). In *A. mellifera*, foragers made more accurate choices when they spent more time making a foraging choice (Burns and Dyer, 2008). Our higher dose TMX-treated bees were faster, but potentially less accurate since they showed no learning (Fig. 1). However, the speed of decision-making did not significantly predict choice accuracy. Only color predicted accuracy, with bees showing significantly more correct choices when yellow was the rewarding color, reflecting innate bee preferences for yellow (Han et al., 2010). It is possible that neonicotinoids can influence speed-accuracy tradeoffs, but determining this requires experiments explicitly designed to test this hypothesis.

4.3. Behavior

Bees fed TMX at low (0.8 ng/bee) or higher (1.34 ng/bee) doses had significantly more abnormal behaviors than control bees. TMX appeared to harm the ability of bees to walk. We observed multiple TMX-fed bees that spent more time on their backs while trying to right themselves. Similarly, Williamson et al. (2014) reported this “upside down” behavior in bees fed TMX in sugar solution. In our study, bees also exhibited higher levels of abnormal behaviors in earlier than later trials. Tosi and Nieh (2017) similarly showed that TMX treated bees had increased falling behavior, greater inability to climb towards the light, and hyperactivity as compared to control bees 1 h after acute exposure and that the effects of TMX on bee behavior changed over time. Within 1 h of an acute dose, bees showed excitation and increased flight duration, distance, and velocity. However, chronic exposure (1 or 2 days) to TMX decreased flight duration, distance, and velocity (Tosi et al., 2017). Many of the abnormal behaviors reported by Tosi et al. (2017) are similar to the ones we observed (Table 1).

5. Summary

We provide the first confirmation, in a visual learning assay, that field-realistic doses of TMX increase the number of abnormal behaviors and the speed of locomotion, inducing apparent hyperactivity. These results raise concerns about the impact of TMX and suggest the need for additional studies on multiple bee species because visual learning plays an important role in all pollinating bees. One of our goals was to improve the visual learning assay developed by Han et al. (2010) for laboratory use. In general, visual learning with restrained bees or bees maintained within the lab is difficult. With restrained bees, Dobrin and Fahrbach (2012) were able to achieve maximum learning at around 40% after five trials. However, if only bees that showed good learning are included, about 70% of bees learned after five trials. We did not filter our data by learning ability, but instead simply eliminated bees that did not complete all of their learning trials. For blue light as the reward, we achieved 75% correct choices with control bees in the final learning trial (trial 6), whereas Han et al. (2010) reported 60% correct choices with control bees in their final learning trial (trial 3). Given our assay modifications, particularly the introduction of a pre-trial, we hoped for even stronger learning. However, our method does advance the techniques for testing visual lab-based learning (Avargues-Weber and Mota, 2016). Further improvements are necessary to reduce learning variation and improve learning before this assay can become sufficiently reliable for standard Risk Assessments.

CRediT authorship contribution statement

Joshua C. Ludicke: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing - original draft, Project administration. **James C. Nieh:** Conceptualization, Methodology, Formal analysis, Resources, Writing - review & editing, Visualization, Supervision, Funding acquisition.

Declaration of competing interest

We, the authors, declare that we have no competing financial, personal, or work interests with people or organizations that could inappropriately bias our work.

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References

Avargues-Weber, A., Mota, T., 2016. Advances and limitations of visual conditioning protocols in harnessed bees. J. Phys. 110, 107–118. https://doi.org/10.1016/j.jphysparis.2016.12.006.

Bass, C., Denholm, I., Williamson, M.S., Nauen, R., 2015. The global status of insect resistance to neonicotinoid insecticides. Pestic. Biochem. Physiol. 121, 78–87. https://doi.org/10.1016/j.pestbp.2015.04.004.

Bitterman, M.E., Menzel, R., Fietz, A., Schäfer, S., 1983. Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). J. Comp. Psychol. 97, 107–119. https://doi.org/10.1037/0735-7036.97.2.107.

Burns, J.G., Dyer, A.G., 2008. Diversity of speed-accuracy strategies benefits social insects. Curr. Biol. 18, R953–R954. https://doi.org/10.1016/j.cub.2008.08.028.

Chittka, L., Dyer, A.G., Bock, F., Dornhaus, A., 2003. Bees trade off foraging speed for accuracy. Nature 424, 388.

Craddock, H.A., Huang, D., Turner, P.C., Quirós-Alcalá, L., Payne-Sturges, D.C., 2019. Trends in Neonicotinoid Pesticide Residues in Food and Water in the United States. pp. 1–16. https://doi.org/10.1186/s12940-018-0441-7. 1999–2015.

Decourtye, A., Henry, M., Desneux, N., 2013. Environment: overhaul pesticide testing on bees. Nature 497https://doi.org/10.1038/497188a. 188–188.

Dietemann, V., Ellis, J.D., Neumann, P., 2013. The COLOSS BEEBOOK Volume I: Standard Methods for *Apis* mellifera Research.

Dobrin, S.E., Fahrbach, S.E., 2012. Visual associative learning in restrained honey bees with intact antennae. PloS One 7, e37666. https://doi.org/10.1371/journal.pone.0037666.

Dukas, R., Visscher, P.K., 1994. Lifetime learning by foraging honey bees. Anim. Behav. 48, 1007–1012.

EFSA, 2012. Statement on the findings in recent studies investigating sub-lethal effects in bees of some neonicotinoids in consideration of the uses currently authorised in Europe. EFSA Journal 10, 1–27. https://doi.org/10.2903/j.efsa.2012.2752.

Fischer, J., Müller, T., Spatz, A.-K., Greggers, U., Grünewald, B., 2014. Neonicotinoids interfere with specific components of navigation in honeybees. PloS One 9, e91364. https://doi.org/10.1371/journal.pone.0091364.s005.

Han, P., Niu, C.-Y., Lei, C.-L., Cui, J.-J., Desneux, N., 2010. Use of an innovative T-tube maze assay and the proboscis extension response assay to assess sublethal effects of GM products and pesticides on learning capacity of the honey bee *Apis mellifera* L. Ecotoxicology 19, 1612–1619. https://doi.org/10.1007/s10646-010-0546-4.

Henry, M., Beguin, M., Requier, F., Rollin, O., Odoux, J.F., Aupinel, P., Aptel, J., Tchamitchian, S., Decourtye, A., 2012. A common pesticide decreases foraging success and survival in honey bees. Science 336, 348–350. https://doi.org/10.1126/science.1215039.

Hung, K.-L.J., Kingston, J.M., Albrecht, M., Holway, D.A., Kohn, J.R., 2018. The worldwide importance of honey bees as pollinators in natural habitats. Proc. Roy. Soc. B 285https://doi.org/10.1098/rspb.2017.2140. 20172140–8.

Johnson, R.M., Ellis, M.D., Mullin, C.A., Frazier, M., 2010. Pesticides and honey bee toxicity–USA. Apidologie. https://doi.org/10.1051/apido/2010018>.

Kah, M., Walch, H., Hofmann, T., 2018. Environmental fate of nanopesticides: durability, sorption and photodegradation of nanoformulated clothianidin. Environ. Sci.: Nano 5, 882–889. https://doi.org/10.1039/C8EN00038G.

Lundin, O., Rundlöf, M., Smith, H.G., Fries, I., Bommarco, R., 2015. Neonicotinoid insecticides and their impacts on bees: a systematic review of research approaches and identification of knowledge gaps. PloS One 10https://doi.org/10.1371/journal.pone.

0136928. e0136928–20.
Matsumoto, Y., Sandoz, J.-C., Giurfa, M., 2012. Revisiting olfactory classical conditioning of the proboscis extension response in honey bees: a step toward standardized procedures. J. Neurosci. Methods 211, 159–167. https://doi.org/10.1016/j.jneumeth.2012.08.018.
Muth, F., Francis, J.S., Leonard, A.S., 2019. Modality-specific impairment of learning by a neonicotinoid pesticide. Biol. Lett. 15https://doi.org/10.1098/rsbl.2019.0359. 20190359–5.
Nauen, R., Ebbinghaus-Kintscher, U., Salgado, V.L., Kaussmann, M., 2003. Thiamethoxam is a neonicotinoid precursor converted to clothianidin in insects and plants. Pestic. Biochem. Physiol. 76, 55–69. https://doi.org/10.1016/S0048-3575(03)00065-8.
Sanchez-Bayo, F., Goka, K., 2014. Pesticide residues and bees – a risk assessment. PloS One 9https://doi.org/10.1371/journal.pone.0094482. e94482–16.
Tison, L., Rößner, A., Gerschewski, S., Menzel, R., 2019. The neonicotinoid clothianidin impairs memory processing in honey bees. Ecotoxicol. Environ. Saf. 180, 139–145. https://doi.org/10.1016/j.ecoenv.2019.05.007.
Tosi, S., Burgio, G., Nieh, J.C., 2017. A common neonicotinoid pesticide, thiamethoxam, impairs honey bee flight ability. Sci. Rep. 7, 1–8. https://doi.org/10.1038/s41598-017-01361-8.
Tosi, S., Nieh, J.C., 2019. Lethal and sublethal synergistic effects of a new systemic pesticide, flupyradifurone (Sivanto ®), on honeybees. Proc. Roy. Soc. B 286, 20190433–20190446. https://doi.org/10.1098/rspb.2019.0433.
Tosi, S., Nieh, J.C., 2017. A common neonicotinoid pesticide, thiamethoxam, alters honey bee activity, motor functions, and movement to light. Sci. Rep. 7, 1–13. https://doi.org/10.1038/s41598-017-15308-6.
Williamson, S.M., Willis, S.J., Wright, G.A., 2014. Exposure to neonicotinoids influences the motor function of adult worker honeybees. Ecotoxicology 23, 1409–1418. https://doi.org/10.1007/s10646-014-1283-x.
Williamson, S.M., Wright, G.A., 2013. Exposure to multiple cholinergic pesticides impairs olfactory learning and memory in honeybees. J. Exp. Biol. 216, 1799–1807. https://doi.org/10.1242/jeb.083931.
Winfree, R., Gross, B.J., Kremen, C., 2011. Valuing pollination services to agriculture. Ecol. Econ. 71, 80–88. https://doi.org/10.1016/j.ecolecon.2011.08.001.
Wright, G.A., Softley, S., Earnshaw, H., 2015. Low doses of neonicotinoid pesticides in food rewards impair short-term olfactory memory in foraging-age honeybees. Sci. Rep. 5, 1–7. https://doi.org/10.1038/srep15322.
Yang, E.-C., Chang, H.-C., Wu, W.-Y., Chen, Y.-W., 2012. Impaired olfactory associative behavior of honeybee workers due to contamination of imidacloprid in the larval stage. PloS One 7, e49472.
Zhang, S.W.E.A., 1996. Maze Learning by Honeybees 1–16.